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# B Cell-Activating Factor Is a Novel Diagnosis Parameter for Asthma

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**Key Words**
B cell-activating factor · Asthma · Immunoglobulin E · Forced expiratory volume in 1 s · T cell · Antigen-presenting cells

**Abstract**
***Background:*** Asthma is a life-threatening immediate-type allergic disease. B cell-activating factor (BAFF) is a key regulator of B lymphocyte development and is required to generate and maintain the mature B cell pool. ***Objectives:*** To investigate the level of BAFF in the serum of asthma patients and the role of BAFF on T cells. ***Methods:*** The BAFF level was measured by enzyme-linked immunosorbent assay. Peripheral blood mononuclear cells (PBMC) from asthma patients were analyzed by flow cytometry. T8.1 cells were used to test the role of BAFF on T cell-antigen-presenting cell (APC) conjugate formation. ***Results:*** The BAFF level in patient serum was elevated relative to normal serum. Immunoglobulin E (IgE) concentration and the percentage of CD3+ T and CD19+ B cells vary according to the serum BAFF level. Patients with high BAFF and high IgE (group II) and those with high BAFF and low IgE (group III) show a high ratio of CD3+ T to CD19+ B cells, and the opposite is seen for patients with low BAFF and high IgE (group I) and those with low BAFF and low IgE (group IV). The addition of BAFF increased PBMC proliferation and T cell-APC conjugate formation. BAFF concentration in serum decreased after treatment with antiasthmatic drugs including glucocorticoids and immunosuppressants. ***Conclusion:*** These findings suggest that the serum BAFF level is high in both IgE-mediated asthma and non-IgE-mediated asthma and extend our knowledge about the fact that BAFF may play a stimulatory role on the proliferation of T cells. Thus, BAFF could be a parameter to monitor the severity of asthma symptoms.



## Introduction

Asthma is a life-threatening immediate-type allergic disease and chronic inflammatory disorder of the airway. Airway inflammation may be acutely and chronically associated with the development of airflow limitation resulting from bronchoconstriction, airway edema or mucus secretion [1]. Immunoglobulin E (IgE) isotype is a critical factor associated with allergic disease, such as asthma, and is involved in host immune defense mechanisms against parasitic infection [2]. Rackeman [3] first introduced the terms 'extrinsic' and 'intrinsic' asthma in 1947. In 1985, Ostergaard [4] classified two types of asthma, extrinsic IgE-mediated and intrinsic non-IgE-mediated asthma. Recently, Corrigan [5] reviewed the possible role of IgE-mediated mechanisms in asthma pathogenesis with respect to intrinsic or nonatopic asthma. Non-IgE-mediated asthma in children showed severe lung inflation. The etiology of non-IgE-mediated asthma, however, has yet to be established.

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B cell-activating factor (BAFF), also known as BLys, TALL-1, zTNF4 or THANK, belongs to the tumor necrosis factor family. BAFF is a key regulator of B lymphocyte development and is required to generate and maintain the mature B cell pool. Its biological role is mediated by the specific receptors, B cell maturation antigen (BCMA), transmembrane activation and calcium modulator and cyclophilin ligand interactor (TACI), and BAFF receptor (BAFF-R) [6–11]. BAFF-deficient mice display a near complete loss of follicular- and marginal-zone B lymphocytes. This suggests that BAFF is necessary to maintain the homeostatic development, differentiation and proliferation of B cells. BAFF-transgenic mice exhibit a breakdown of B cell tolerance, leading to a systemic lupus erythematosus (SLE)-like condition [12, 13]. BAFF levels are high in human serum from patients with autoimmune diseases such as rheumatic arthritis, autoimmune diabetes, Sjögren's syndrome and multiple sclerosis [14–18].

BAFF activity was observed on naive as well as on effector/memory T cells (both CD4+ and CD8+ subsets) [19]. Recombinant BAFF induces thymidine incorporation and cytokine secretion in T cells by stimulating the T cell receptor (TCR) [11, 19, 20]. BAFF-R is able to deliver a complete costimulatory signal to T cells [19]. Bacterial products upregulate BAFF production in T cells, and a low level of BAFF transcription, upregulated upon TCR stimulation, has also been detected in T cells [11, 19]. BAFF may regulate T cell immunity during antigen-presenting cell (APC)-T cell interactions and function as an autocrine factor once T cells have detached from the APC [11]. Previous data have shown that T cell proliferation secondary to BAFF is mediated entirely by BAFF-R and not by TACI [20].

Based on the different immunopathological mechanisms existing between autoimmune diseases and allergic diseases, such as asthma, we analyzed the serum BAFF concentration using the enzyme-linked immunosorbent assay (ELISA) and the changes in the cellular phenotype, CD3 or CD19 of immune cells from asthma patients using flow cytometry. We also investigated the effect of BAFF on T cell population.

## Materials and Methods

*Patient Selection*

After IRB-approved informed consent, blood was drawn in heparinized tubes for the purification of peripheral blood mononuclear cells (PBMC) and in nonheparinized tubes for the separation of serum from patients with respiratory function-verified asthma who were either untreated or at least 1 month postchemotherapy. Patients with active infections or other serious medical conditions were not included in this study.

*Reagents*

The following reagents were obtained from commercial sources. Anti-human BAFF antibodies, biotin-labeled anti-human BAFF antibodies and streptavidin-labeled horseradish peroxidase were purchased from R&D Systems (Minneapolis, Minn., USA). PE-conjugated anti-CD3 antibodies and FITC-conjugated anti-CD19 antibodies were purchased from BD Biosciences (San Jose, Calif., USA). TACI:Fc proteins were purchased from Alexis (Carlsbad, Calif., USA).

*Cell Purification and Cultures*

T8.1 and L625.7 cells were kindly provided by Dr. Acuto (Pasteur Institute, Paris, France), and maintained following a previous report [23]. Patient peripheral blood samples were collected and PBMC cells were isolated by density gradient centrifugation over Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo., USA). Cells were cultured in the presence or absence of phytohemagglutinin (PHA) in RPMI 1640 media (Gibco, Rockville, Md., USA) supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 10 mM Hepes (pH 7.4), 100 U/ml penicillin, and 100 units/ml streptomycin (Sigma Chemical Co., St. Louis, Mo., USA) at 37°C and 5% $CO_2$ in air. Cells were pulsed with 1 μCi/well of [$^3$H]-thymidine (113 Ci/nmol, NEN, Boston, Mass., USA) for 4–6 h and collected on nitrocellulose filter with automated cell harvester (Inotech, Dottikon, Switzerland). Then, the amount of [$^3$H]-thymidine incorporated into cells was measured as cpm by a Wallac Microbeta scintillation counter (Wallac, Turku, Finland).

*ELISA for BAFF and IgE*

BAFF or IgE concentration was measured by ELISA according to the following method: 100 μl of anti-BAFF (R&D System) or anti-IgE antibodies (Bethyl Laboratories, Montgomery, Tex., USA) diluted in diluent [1% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4] were transferred to an ELISA plate and incubated overnight at room temperature. The plate was washed twice with washing buffer (0.05% Tween 20 in PBS, pH 7.4) and blocked with blocking solution (1% bovine serum albumin, 5% sucrose and $NaN_3$ in PBS) for 1 h, then washed twice with washing buffer. Serum from asthma patients was added to the anti-BAFF antibody-coated ELISA plate. After 2 h incubation, the plate was washed twice and biotin-conjugated anti-BAFF or anti-IgE antibodies (R&D System) were added and then incubated for 2 h at room temperature. After washing the plate twice, streptavidin-conjugated horseradish peroxidase (R&D System) was added and incubated for 20 min. Substrate for horseradish peroxidase was a 1:1 mixture of color reagent A ($H_2O_2$) and color reagent B [tetramethylbenzidine (TMB); R&D System]. After 20 min incubation, 50 μl of 1 M $H_2SO_4$ was added to stop the reaction. Absorbance was measured at 450 nm.

*Flow Cytometry Analyses*

PBMC (1–10 × $10^5$) were suspended in 2% FCS containing Hanks' balanced salt solution (HBSS) and incubated with PE-conjugated anti-CD3 or FITC-conjugated anti-CD19 antibodies for 20 min on ice. Cells were washed with HBSS and analyzed by CELLQuest™ software in FACScalibur™ (Becton Dickinson).

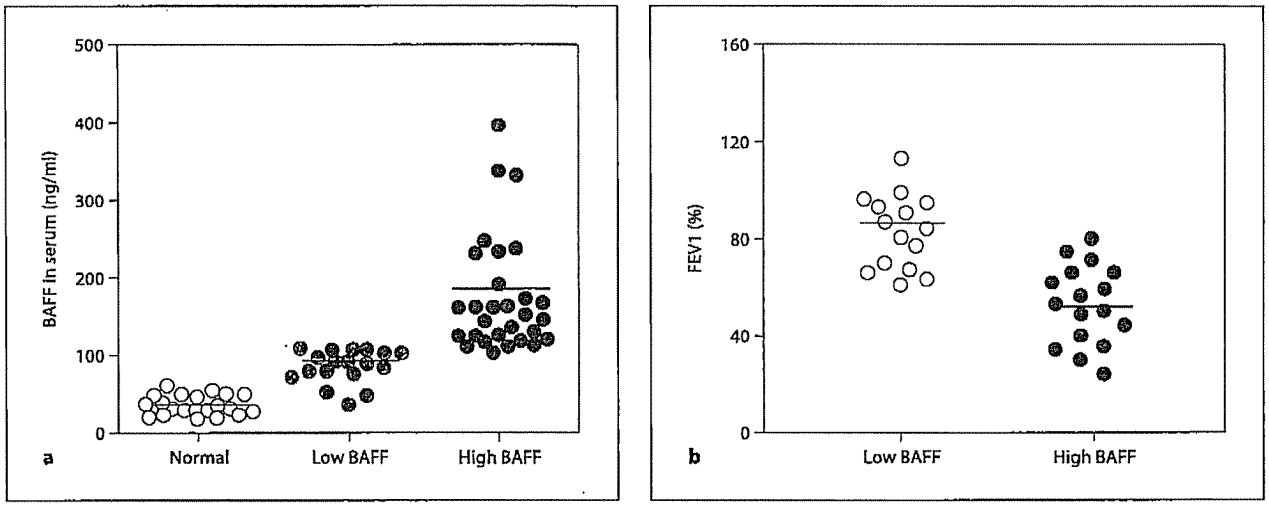


**Fig. 1.** The IgE concentration varies according to the serum BAFF level. **a** Serum samples were collected 3 times. A total of 103 asthmatic patients were recruited. BAFF levels were measured by ELISA. Then, patients were grouped into two levels of BAFF concentration: low (above normal and less than 100 ng/ml) and high (above 100 ng/ml). Control is a normal serum BAFF level, approximately 40 ng/ml. Assays were performed 3 times with serum samples from three different hospitals. **b** FEV1 (%) in the group with a high BAFF level was lower than in the group with a low BAFF level. Horizontal bars indicate the mean for BAFF level or FEV1 (%).

*RT-PCR*

RNA was isolated from PMBC using TRIzol (Invitrogen, Carlsbad, Calif., USA). cDNA was synthesized from 1 μg of total RNA, using oligo $dT_{18}$ primers and superscript reverse transcriptase in a final volume of 21 μl (Bioneer, Taejeon, Korea). For standard PCR, 1 μl of the first-strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer). PCR amplification proceeded as follows: 33 thermocycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, using oligonucleotides specific for BAFF (sense: aatactaataggacaggg, antisense: tccacgaggagcactt), BAFF-R (sense: gcaatcagaccgagtgcttcg, antisense: ctatgctctgggccagctgt), TACI (sense: atggctatggcattctgccc, antisense: tcagatccctggtgccttcc), BCMA (sense: tctttcagtgatccagtccc, antisense: cactttgcaaagcagttggc) and β-actin (sense: tggaatcctgtggcatccatgaaac, antisense: taaaacgcagctcagtaacagtccg).

*APC-T Cell Conjugate Formation*

Assays were performed by a previously reported method [21]. Briefly, antigen-induced T cell adhesion was quantified by using a colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] [22]. Confluent L625.7 cells ($2 \times 10^5$) were cultured overnight with tetanus $toxin^{830-843}$ oligopeptide (Ttox 830–843) at a final concentration of 1 μg/ml in flat-bottom 24-well plates in triplicate (Falcon, Oxford, UK). T8.1 cells ($1 \times 10^6$) were added to a final volume of 1.0 ml. Plates were then incubated at 37°C in 5% $CO_2$ for 45 min. We removed unbound T8.1 cells by washing the plates 3 times with prewarmed medium. The remaining adherent cells were then incubated with 50 μg/ml of MTT at 37°C for 2 h. The amount of T8.1 cell adhesion to L625.7 cells was calculated by subtracting the background signal obtained with L625.7 cells alone. We determined the percentage of T8.1 cells that bound by comparing values obtained for background-subtracted T8.1 binding to the signal for the total number of added T8.1 cells. The values for the MTT assay are expressed as an optical density reading at 595 nm.

*Statistical Analyses*

Experimental differences were tested for statistical significance using ANOVA and Student's t test. A p value of $<0.05$ or $<0.01$ was considered to be significant.

## Results

### *BAFF Concentration Is Increased in Asthma Patient Serum*

BAFF transgenic mice tend to have autoimmune diseases, such as SLE; furthermore, patients with rheumatic arthritis have higher serum levels of BAFF [12, 13]. These diseases represent Th1-mediated inflammatory diseases. However, the relationship between BAFF and Th2-mediated diseases, such as asthma, has so far not been studied. Usually, patients with Th2-mediated allergic asthma have a high level of serum IgE. BAFF enhances class switch DNA recombination to produce IgE [10]. Thus, we analyzed the BAFF level in serum samples from asthma patients using ELISA. The control BAFF level was about

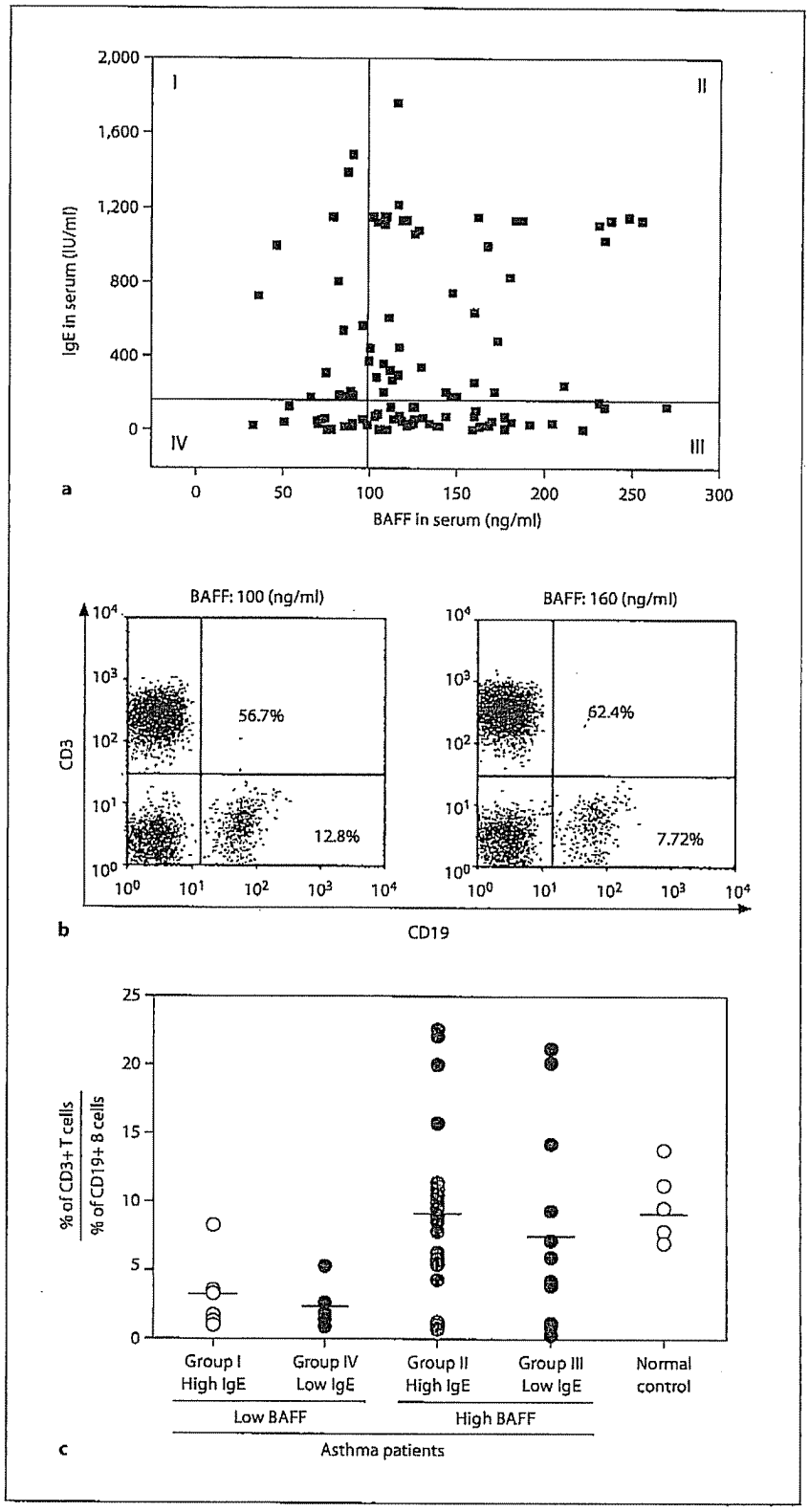


**Fig. 2.** IgE concentration and CD3 or CD19 expression vary according to the serum BAFF level. **a** BAFF and IgE levels in 103 asthmatic patient sera were measured by ELISA. Each point represents a different serum sample. Samples were grouped into four categories: low BAFF concentration and high IgE concentration (I), high BAFF concentration and high IgE concentration (II), high BAFF concentration and low IgE concentration (III) and low BAFF concentration and low IgE concentration (IV). **b** PBMC were prepared from blood samples of asthmatic patients and stained with PE-conjugated anti-CD3 and FITC-conjugated anti-CD19 antibodies. Then, cells were analyzed by flow cytometry. **c** The CD3+ T to CD19+ B cell ratio of each patient was plotted for groups I, II, III and IV according to the levels of BAFF and IgE in figure 1b. Horizontal bars indicate the mean for T to B cell ratio.

40 ng/ml, but the BAFF level was above control in asthma patients (fig. 1). Patients were grouped into two levels of BAFF concentration: low (above normal and no more than 100 ng/ml) and high (more than 100 ng/ml). The percentage of forced expiratory volume in 1 s (FEV1) was reduced by the increase of the serum BAFF level. FEV1 (%) in the group with a high BAFF level was lower than in the group with a low BAFF level (fig. 1b). Clinically, other asthma symptoms were less severe in the patients with a low BAFF level (clinical symptom, data not shown). Thus, the BAFF level might be a useful gauge of the severity of asthma symptoms. These data suggest that the serum BAFF level might be a useful parameter to monitor asthma symptoms.

*BAFF versus IgE, CD3+ T and CD19+ B Cells*

We determined the levels of the classic indicators of asthma, namely IgE, for each patient (fig. 2a). We classified patients into four groups: group I: high IgE and low BAFF; group II: high IgE and high BAFF; group III: high BAFF and low IgE; group IV: low BAFF and low IgE. Asthma patients usually have a high airway hyperresponsiveness independent of IgE level. This condition was confirmed by the fact that the BAFF level correlated with FEV1 (%) (fig. 1b) and PC20 (data not shown). This suggests that BAFF represents an additional indicator that can explain the airway hyperresponsiveness in non-IgE-mediated asthma.

To study changes in immune cell population in asthma, we purified mononuclear cells from peripheral blood samples taken from asthma patients. B and T cells were incubated with anti-CD19 and anti-CD3 antibodies, respectively. CD3+ T cells and CD19+ B cells were analyzed by flow cytometry. Percentages of T and B cells vary following BAFF level changes (data not shown). Patients with a low BAFF level had a low T to B cell ratio, and the opposite was evident for patients with a high BAFF level (fig. 2b). The CD3+ T to CD19+ B cell ratio of each patient was plotted for groups I, II, III and IV according to the levels of BAFF and IgE. The ratio of CD3+ T cells to CD19+ B cells was dependent on the BAFF level, but it was independent of serum IgE level (fig. 2c). These data suggest that BAFF might activate T cell proliferation and asthma may be regulated by BAFF-induced T cell activation.

*Effect of BAFF on T Cell Activation*

To confirm the role of BAFF in asthma patients on the increase of T cell ratio, we purified PBMC from peripheral blood from normal volunteers. Cells were treated with 50 or 100 ng/ml of BAFF and then incubated for 48 h. As shown in figure 3, BAFF stimulated T cell proliferation in the presence or absence of PHA costimulation. As PBMC comprise the majority of T cells, PBMC proliferation can be interpreted as T cell proliferation. These data are consistent with the increased T to B cell ratio following the increase in serum BAFF (fig. 2b).

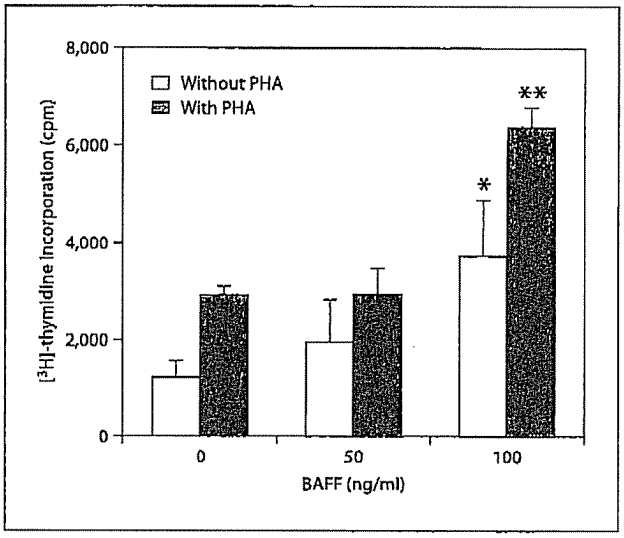


**Fig. 3.** T cell proliferation was increased by BAFF. PBMC were prepared from healthy volunteer donor blood. Cells were stimulated with PHA in the presence or absence of 50 or 100 ng/ml BAFF for 48 h. Cells were pulsed with 1 μCi [$^3$H]-thymidine for 6 h and collected with a cell harvester. Radioactivity was measured with a β-scintillation counter. Data in bar graphs represent mean ± SED. * $p < 0.05$; ** $p < 0.01$, significantly different from BAFF nontreated control at the same experimental conditions.

*APC-T Cell Conjugate Formation by BAFF*

To reaffirm the function of BAFF on T cell, we studied APC-T cell conjugate formation-mediated T cell activation. We used T8.1 as T cells and L625.7 as APCs [23]. T8.1 cells expressed BAFF-R and TACI (fig. 4a). When L625.7 cells were stimulated with Ttox 830–843 as antigen peptide, BAFF expression increased time-dependently (fig. 4b). T8.1 cells were pretreated with BAFF at the concentrations of 20 or 200 ng/ml and incubated with Ttox 830–843-stimulated L625.7 cells for 45 min. Figure 4c showed that APC-T cell conjugate formation increased BAFF dose-dependently. As APC-T cell conjugate formation was also increased only in the presence of Ttox 830–843, it implicates that BAFF produced by Ttox 830–843 stimulation on L625.7 cells induced APC-T cell conjugate formation. These data were proved by which BAFF-medi-

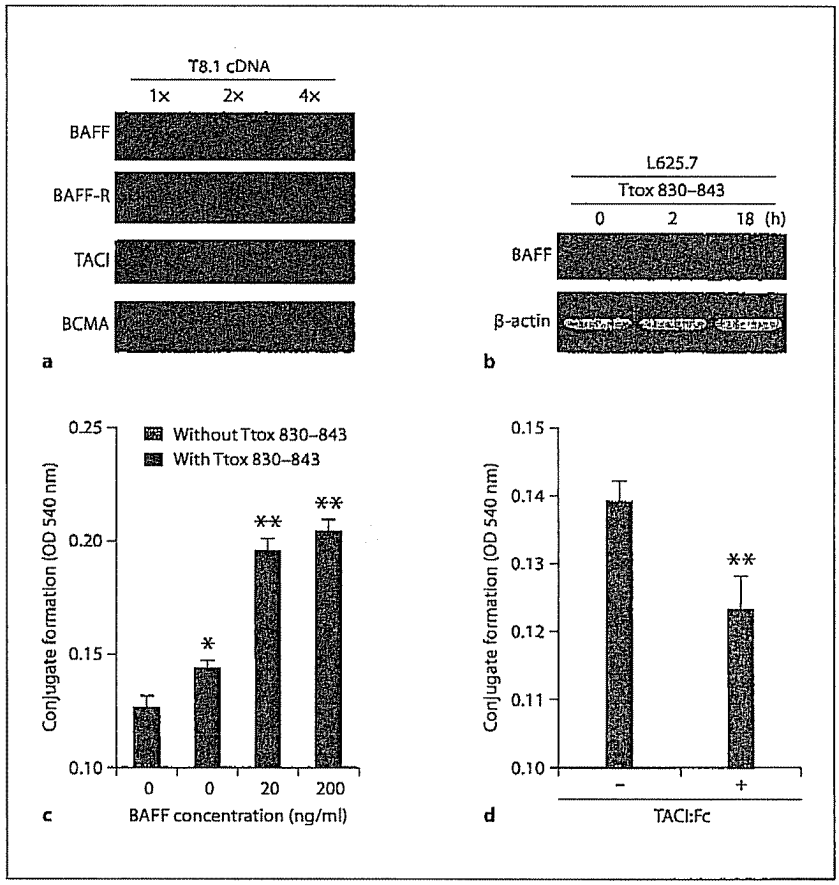


**Fig. 4.** APC-T cell conjugation was increased by pretreatment of T8.1 cells with BAFF. **a** BAFF-R, BCMA and TACI expression was detected by RT-PCR in samples containing 1-, 2- and 4-fold levels of cDNA from T8.1 cells. **b** Soluble BAFF expression was detected by RT-PCR in samples prepared at 2 and 16 h after Ttox 830–843 stimulation of L625.7 cells. β-Actin was used as a quantitative control of RT-PCR. **c** T8.1 cells were pretreated with BAFF and L625.7 cells were stimulated with Ttox 830–843 for 16–18 h. APC-T cell conjugate formation was accomplished by the incubation of T8.1 and L625.7 cells for 45 min. Conjugate formation was quantified with MTT assay. **d** APC-T cell conjugate formation was reduced by the incubation with TACI:Fc (25 μg/ml). T8.1 and Ttox 830–843 oligopeptide-stimulated L625.7 cells were incubated in the presence or absence of TACI:Fc for 45 min. Conjugate formation was quantified with MTT assay. Data in bar graphs represent mean ± SED. * $p < 0.05$; ** $p < 0.01$, significantly different from Ttox 830–843 oligopeptide-treated control at the same experimental conditions.

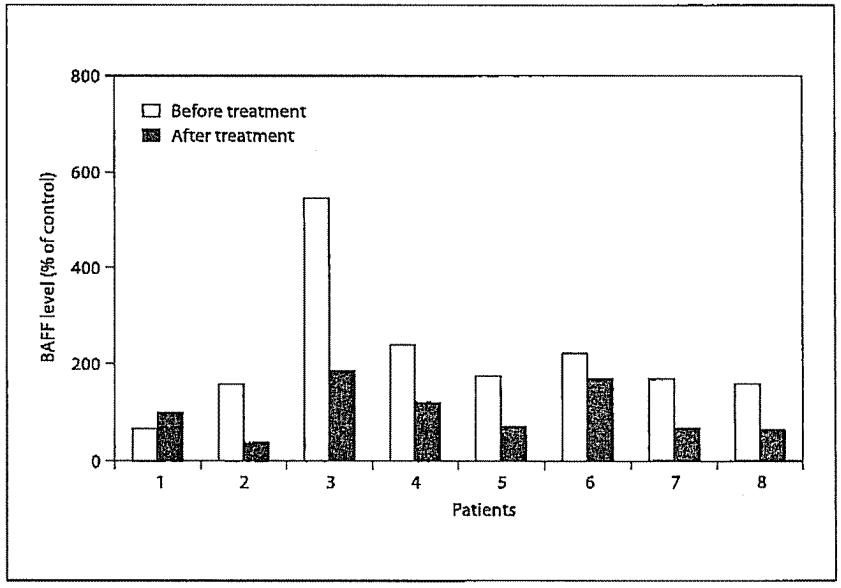


**Fig. 5.** The serum BAFF level was correlated with the severity of asthmatic symptoms. It was reduced after the treatment with the antiasthmatic agent. Serum samples were collected before and 3–6 months after the treatment with the antiasthmatic agents from the same patients. BAFF levels were measured by ELISA.

Table 1. Antiasthmatic agents for the treatment of asthma patients

| Patients | Antiasthmatic agent |
|---|---|
| 1 | Inhaled steroid, β-agonist, antihistamine |
| 2 | Inhaled steroid, β-agonist, antileukotriene |
| 3 | Inhaled steroid, β-agonist, antileukotriene |
| 4 | Inhaled steroid, β-agonist |
| 5 | Inhaled steroid, β-agonist, antileukotriene, antihistamine |
| 6 | Inhaled steroid, antileukotriene, antihistamine |
| 7 | Inhaled steroid, β-agonist, antileukotriene |
| 8 | Inhaled steroid, β-agonist, antileukotriene, antihistamine |

ated APC-T cell conjugate formation was attenuated by the incubation with TACI:Fc, BAFF scavenger (fig. 4d). This supports the data that BAFF stimulated T cell proliferation in figure 3.

*BAFF Level Decreased after the Treatment with Antiasthmatic Drugs*

Serum samples were collected from the same patient before and 3–6 months after the treatment with antiasthmatic drugs (table 1). The BAFF level of 8 patients was measured by ELISA and compared before and after the treatment. Serum BAFF level in the same patients was lowered by the treatment with antiasthmatic drugs (fig. 5). Therefore, it is suggested that BAFF could be a novel parameter to monitor the severity of asthma symptoms.

## Discussion

BAFF is a B cell survival factor during development. BAFF-deficient mice have a defect in B cell development [13], and BAFF-transgenic mice have autoimmune diseases, such as SLE, resulting from an excess number of B cells [12]. Autoimmune diseases are usually interpreted as Th1-mediated immune disorders. However, asthma is an allergic disease mediated by the production of various cytokines, such as IL-4 and IL-10, from Th2 cells. In this study, we determined the BAFF level in the sera of asthma patients. Asthma patients have an elevated BAFF level. This result is almost identical in three separate experiments. BAFF functions in class switch DNA recombination to produce IgE [10, 11, 24]. Here, we found that the BAFF level was high even in sera of asthma patients with low IgE concentration. These data suggest that the serum BAFF level could be an additional parameter to classical parameters to monitor the asthma symptoms. In the mean time, a recent study with a mouse model for asthma showed that BAFF-transgenic mice suppressed allergic airway inflammation and Ag-specific T cell responses [25]. Thus, data also suggest that the high BAFF level in patients with asthma could be secondarily produced to suppress the inflammation.

Some patients had a low IgE level but still showed high airway sensitivity like those in group III (fig. 2a). Until now, there has been little explanation for such symptoms in asthma patients. With our data, asthma in group III patients could be explained and diagnosed by a high BAFF level in serum. This suggests that BAFF is a novel indicator that can explain the airway hyperresponsiveness in non-IgE-mediated asthma. Thus, future studies must address the detailed mechanism of action in BAFF-mediated allergic reaction.

A high BAFF level in serum was reflected in various percentages of CD3+ T and CD19+ B cells (fig. 2b). We demonstrated that patient group II, having high BAFF and high IgE levels, had a high CD3+ T to CD19+ B cell ratio. Patient group III, having high BAFF and low IgE, also had a high CD3+ T to CD19+ B cell ratio (fig. 2c). These results suggest that BAFF affects T cell activation in both IgE-mediated and non-IgE-mediated asthma and regulates IgE production through BAFF-mediated T cell activation.

BAFF activity was observed on naive as well as on effector/memory T cells [19]. Recombinant BAFF induces T cell functions, suboptimally, through the TCR [11, 19, 20]. A low level of BAFF transcription, upregulated upon TCR stimulation, was detected in T cells [11]. BAFF may regulate T cell immunity during APC-T cell interactions and act as an autocrine factor once T cells have detached from the APC [11]. In addition, BAFF-transgenic mice showed an increased T cell number and expression of BAFF in T cells [12, 26]. The fact that high BAFF levels are correlated with a high ratio of CD3+ T to CD19+ B cells implies that the elevated BAFF level may play a role like a costimulatory molecule in TCR-stimulated T cell activation [11, 19, 20, 24].

TACI and BAFF-R expression in T cells [24] was confirmed in T8.1 cells (fig. 4a). Our data showed that APC-T cell conjugate formation increased with the pretreatment of T8.1 cells with BAFF and the antigenic stimulation of L625.7 cells with Ttox 830–834 (fig. 4c) that induces soluble BAFF expression (fig. 4b). This was attenuated by the treatment with TACI:Fc (fig. 4d). In fact, previous studies have shown that T cell proliferation secondary to BAFF is mediated entirely by BAFF-R, not TACI [24]. However, the functions of BAFF receptors,

BAFF-R and TACI, have not yet been defined but it is expected that they function differently in T cells. Our data suggest that CD3+ T cells may communicate with either APCs or CD19+ B cells through BAFF-R or TACI in which BAFF may act as a communication bridge between T and B cells or APCs.

Research thus far indicates that BAFF signaling pathways are mediated through the regulatory subunit gamma of the IκB kinase (NEMO)-independent NF-κB activation and PKC-δ and PLC-γ [27–29]. Although the BAFF-mediated signaling pathways have not been fully identified, BAFF is expected to play a role in airway hyperresponsiveness mediated by T cells. In conclusion, our data suggest that BAFF may be an effective additional indicator to monitor the severity of asthma symptoms.

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